Effects of Topical Antiglaucoma Medications on Corneal Epithelium as Evaluated by Gene Expression Patterns

Yuka Okada, MD, PhD

Purpose: To examine the expression pattern of the stress-related genes c-fos and c-jun, which encode the 2 major components of activator protein (AP)-1, and cyclooxygenase (COX)-2 in rat corneal epithelium treated with topical antiglaucoma medications and benzalkonium chloride (BAK) preservative.

Methods: Eighty-eight male Wistar rats were used. We instilled antiglaucoma eye drops (0.5% Timoptol, 0.005% Xalatan, or 0.12% Rescula), their chemical constituents (active ingredients), or BAK preservative (0.005%, 0.01%, or 0.02%) in 1 eye of each rat. Fellow eyes served as controls. The eyes were enucleated after various intervals. In situ hybridization and immunohistochemistry were used to detect expression of c-fos, c-jun, and COX-2.

Results: Expression of c-fos, c-jun, and COX-2 was minimally observed in uninjured rat corneal epithelium. Thirty minutes to 1 hour after applying the antiglaucoma eye drops, signals for c-fos and c-jun mRNA were detected in the corneal epithelium. Ninety minutes after applying 0.005% Xalatan, 0.12% Rescula, or their chemical constituents, but not 0.5% Timoptol, COX-2 was detected in corneal epithelium. Expression of c-fos and c-jun seemed more marked with prostaglandins than with timolol. Thirty minutes to 1 hour after instillation of 0.02% BAK preservative, signals for c-fos and c-jun mRNA were detected in the corneal and conjunctival epithelium. COX-2 was not induced by 0.5% Timoptol or BAK preservative. COX-2 mRNA was not affected by applying 0.005% or 0.01% BAK preservative. Proteins of these components were also detected, indicating that each mRNA expression was followed by protein synthesis.

Conclusions: Corneal and conjunctival epithelial cells are transcriptionally activated transiently at an early phase after topical administration of antiglaucoma medications and BAK preservative. Stimulatory effects of prostaglandin drugs on corneal epithelial cells were more marked than those with timolol. Expression of COX-2 may potentially be involved in inflammatory response in the corneal epithelium.
expressed in many tissues. In contrast, COX-2, which is normally expressed at low levels, is potently induced by proinflammatory agents.\textsuperscript{17–19}

Previously, we showed that mRNA encoding AP-1 components and COX-2 is transiently upregulated in healing corneal epithelium after epithelial debridement.\textsuperscript{20–22} In this study, we examined the expression pattern of AP-1 and COX-2 in rat corneal epithelium treated with antiglaucoma eye drops and BAK preservatives.

\section*{MATERIALS AND METHODS}

\subsection*{Investigational Agents}

All experiments were performed in accordance with the ARVO Resolution on the Use of Animals in Research. Adult male Wistar rats (n = 88) were used. In 1 eye of each rat, the following were instilled: antiglaucomatous eye drops (0.5\% timolol, Timoptol; 0.005\% latanoprost, Xalatan; or 0.12\% isopropyl unoprostone, Rescula), the chemical constituents (active ingredients) of Xalatan (0.005\% latanoprost) and Rescula (0.12\% unoprostone), or BAK preservative at a concentration of 0.005\%, 0.01\%, or 0.02\%. The affected eyes were allowed to heal for 15, 30, 60, 90, 120, and 180 minutes after treatment and then were enucleated. Fellow eyes served as controls. Eyes from untreated rats were also enucleated.

\subsection*{In Situ Hybridization for mRNA of AP-1 Components}

In situ hybridization was carried out by using $^{35}$S-radiolabeled oligonucleotide probes as previously reported.\textsuperscript{20,23} Probes for detection of c-fos and c-jun mRNA were complementary to the nucleotides spanning amino acids 1 to 15 of rat c-Fos protein,\textsuperscript{24} the last 20 amino acids of the predicted c-Jun,\textsuperscript{25} or 1421 to 1460 of rat COX-2 mRNA\textsuperscript{26} sequences. A computer-assisted homology search revealed no identical sequences in any genes in the database. The eyes were embedded in optimal cutting temperature (OCT) compound and quickly frozen. Cryosections (5 mm) were fixed with 4\% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 minutes, rinsed 3 times in 2\times SSC, and dehydrated through a graded ethanol series. Hybridization was performed as described previously\textsuperscript{15} by incubating sections
with hybridization buffer containing each probe (0.5–1.0 × 10^6 cpm/slide) for 16 hours at 37°C. After hybridization, sections were rinsed in 1× SSC at 55°C for 4 × 15 minutes. The sections were dehydrated and coated with Ilford G-5 emulsion diluted 1:1 with water. The sections were dried in a level position for 30 minutes and exposed in tightly sealed dark boxes at 4°C for 8 weeks. After being developed in D-19 (Kodak, Rochester, NY) developer, fixed with photographic fixer, and washed with tap water, the sections were counterstained with 1% neutral red (sodium acetate buffer).

Incubation of sections with a mixture of each 35S-labeled probe and 100-fold excess of respective cold oligonucleotide resulted in no detectable signals.

Immunostaining for AP-1 Component Proteins

Immunohistochemistry for c-Fos and COX-2 proteins was carried out as previously reported.20–22 In brief, the eyes were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hours. Cryosections were incubated with primary antibodies diluted (1:200; c-Fos; Oncogene Science, Cambridge, MA; and 1:200; COX-2, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 24 hours. After washing in PBS, sections were incubated with biotinylated anti–rabbit immunoglobulin G (IgG) or anti–goat IgG (1:250; Vectastain ABC kit; Vector, Burlingame, CA) at room temperature for 1 hour and then with avidin-biotin peroxidase complex (Vectastain ABC kit; Vector) at room temperature for 1 hour. Peroxidase reaction product in tissue sections was visualized by using diaminobenzidine. After counterstaining with methyl green, sections were dehydrated and mounted in balsam. Sections incubated with normal rabbit or goat serum (1:200) and processed in the same way as described above showed no immunostaining in the corneal epithelium.

RESULTS

In Situ Hybridization

Expression of c-fos, c-jun, and COX-2 was minimally observed in uninjured rat corneal epithelium (Figs. 1–3). Thirty minutes to 1 hour after applying antiglaucoma medications, signals for c-fos and c-jun mRNA were detected in the corneal epithelium (Figs. 1, 2). Ninety minutes after applying 0.005% Xalatan, 0.12% Rescula, or these chemical...
constituents, COX-2 was detected in corneal and conjunctival epithelium (Figs. 3–5). Expression of c-fos and c-jun seemed more marked with prostaglandins than with Timoptol. Thirty minutes to 1 hour after instillation of 0.005% Xalatan and 0.12% Rescula, but not 0.5% Timoptol, COX-2 was detected in corneal epithelium. Bar = 100 mm. E, epithelium.

FIGURE 3. Dark-field photomicrographs showing expression of COX-2 mRNA in the corneal epithelium after instillation of antiglaucoma eye drops. Ninety minutes after applying 0.005% Xalatan and 0.12% Rescula, but not 0.5% Timoptol, COX-2 was detected in corneal epithelium. Bar = 100 mm. E, epithelium.

Immunohistochemistry

No immunoreaction for c-Fos and COX-2 was detected in the healthy corneal epithelium (Figs. 7, 8). c-Fos protein (Figs. 3, 6). Signals for this mRNA were not affected by applying BAK preservative at concentrations of 0.005% or 0.01% (Fig. 6).

Conjunctiva

FIGURE 4. Dark-field photomicrographs showing expression of c-fos, c-jun, and COX-2 mRNA in the conjunctival epithelium after instillation of antiglaucoma eye drops. Bar = 100 mm.
was detected in corneal and conjunctival epithelium 120 minutes after applying antiglaucoma medications (Figs. 7, 9). COX-2 protein was detected in corneal and conjunctival epithelium 120 minutes after instillation of 0.005% Xalatan, 0.12% Rescula, and their chemical constituents (Figs. 8, 9). c-Fos protein was also detected in the conjunctival epithelium 120 minutes after instillation of 0.02% BAK preservative (Fig. 10). COX-2 protein was not detected in corneal and conjunctival epithelium after instillation of BAK preservative.

**DISCUSSION**

Corneal and conjunctival epithelial cells are transcriptionally activated transiently at an early phase after instillation of antiglaucoma eye drops and BAK preservative.

Expression of AP-1 was more marked after instillation of PGs than with timolol; 0.005% Xalatan and 0.12% Rescula, and their chemical constituents (Figs. 7, 9), c-Fos protein was also detected in the conjunctival epithelium 120 minutes after instillation of 0.005% latanoprost and 0.12% unoprostone. c-Fos, c-Jun, and COX-2 mRNA was detected in the corneal and conjunctival epithelium after instillation of BAK preservative.

**FIGURE 5.** Dark-field photomicrographs showing expression of c-fos, c-jun, and COX-2 mRNA in the corneal and conjunctival epithelium after instillation of chemical constituents (active ingredients) 0.005% latanoprost and 0.12% unoprostone. c-fos, c-jun, and COX-2 mRNA was detected in the corneal and conjunctival epithelium after instillation. Bar = 100 mm.

activity in organ cultured bovine cornea in a dose-dependent manner.\(^{35}\) These findings suggest that high concentrations of BAK are detrimental to corneal and conjunctival epithelium, and glaucoma medications with low levels of or no BAK are consequently more benign to the ocular surface.

Increased numbers of inflammatory cells and fibroblasts have been described in the conjunctiva and Tenon capsule after long-term topical antiglaucoma medication. Indeed, antiglaucoma eye drops containing BAK stimulated an exaggerated myofibroblast response.\(^{30-33}\) Moreover, BAK was found to be irreversibly toxic to cultured human Tenon capsule fibroblasts at clinically used concentrations. Fibroblast proliferation was higher after treatment with antiglaucoma eye drops with BAK preparation, indicating that the tissue was able to recuperate from the initial injury.\(^{34}\) Long-term preoperative therapy has been reported to have a deleterious effect on surgical outcome by altering postoperative wound healing.\(^{35,36}\)

In humans, the volume of tear fluid accumulated in the conjunctival sacs is \(\sim 7\) mL, and tear production is \(\sim 1.2\) mL/min.\(^{37}\) However, dilution of antiglaucoma eye drops and BAK by tear fluid is greatly reduced in patients with dry eye. Therefore, there is potential a greater risk of adverse events caused by these drugs in patients with dry eye.

AP-1 is rapidly induced in cells on exposure to a wide variety of extracellular stimuli. PGF\(_2\alpha\) has been shown to increase protein kinase C (PKC) activity and PKC translocation in luteal cells.\(^{38-40}\) PKC is involved in PGF\(_2\alpha\)-induced activation of the Raf/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling cascade.
in luteal cells, because the response to PGF$_{2\alpha}$ was mimicked by PKC activator phorbol 12-myristate 13-acetate (PMA). PGF$_{2\alpha}$ and PMA stimulated c-fos and c-jun mRNA expression in primary cultured bovine luteal cells.

In vitro studies have shown that latanoprost may increase PGE$_2$ release in iris and ciliary muscles. COX-2 is strongly induced by proinflammatory agents. Induction of COX-2 expression by PGs, in particular with respect to the cAMP-elevating PGE$_2$, has been extensively studied. In the case of PGF$_{2\alpha}$, COX-2 induction has been observed in corpus luteum of different species. COX-2 might be involved in inflammatory response in the corneal epithelium after instillation of 0.005% Xalatan, 0.12% Rescula, or their chemical constituents.

We previously showed that mRNA encoding AP-1 components and COX-2 is transiently upregulated in healing the corneal epithelium after epithelial debridement and ethanol exposure. The expression pattern after antiglaucoma eye drops was similar to that after ethanol exposure. Such events in the epithelium are believed to be unfavorable in maintenance of tissue homeostasis. Therefore, upregulation of these components on exposure to antiglaucoma eye drops or their constituents including preservatives might suggest undesirable stimuli to the cells in the epithelium.

In the final analysis, all aspects of the potential benefits and detriments caused by antiglaucoma eye drops (and their preservatives) on corneal and conjunctival epithelium should be taken into consideration when prescribing these agents.

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FIGURE 7. Protein expression of c-Fos in corneal epithelium after instillation of antiglaucoma eye drops. No immunoreaction for c-Fos was detected in control epithelium. c-Fos protein was detected in the corneal epithelium 120 minutes after applying antiglaucoma medications. Bar = 100 mm. E, epithelium.

FIGURE 8. Protein expression of COX-2 in corneal epithelium after instillation of antiglaucoma eye drops. No immunoreaction for COX-2 was detected in control epithelium. COX-2 protein was detected in the corneal epithelium 120 minutes after applying 0.005% Xalatan and 0.12% Rescula but not 0.5% Timoptol. Bar = 100 mm. E, epithelium.
REFERENCES


FIGURE 9. Protein expression of c-Fos and COX-2 in corneal epithelium after instillation of chemical constituents 0.005% latanoprost and 0.12% unoprostone. No immunoreaction for c-Fos and COX-2 was detected in control epithelium. c-Fos and COX-2 proteins were detected in the corneal epithelium 120 minutes after applying 0.005% Xalatan and 0.12% Rescula but not 0.5% Timoptol. Bar = 100 mm. E, epithelium.

FIGURE 10. Protein expression of c-Fos in the conjunctival epithelium after instillation of BAK preservative. c-Fos protein was detected in the conjunctival epithelium 120 minutes after applying 0.02% BAK preservative. Bar = 100 mm.
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